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(54) Title: EXPRESSION SYSTEM

(57) Abstract: An immunogenic reagent which produces an immune response which isprotective against *Bacillus anthracis*, said reagent compr ising one or more polypeptides which together represent up tothree domains of the full length Protective Antigen (PA) of *B . anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof.The polypeptides of the immunogenic reagent as well as full length PA are produced by expression from *E. coli*. High yields of polypeptide are obtained using this method. Cells,vectors and nucleic acids used in the method are also describedand claimed.

Expression System

The present invention relates to polypeptides which produce an immune response which is protective against infection by *Bacillus anthracis*, to methods of producing these, to recombinant *Escherischia coli* cells, useful in the methods, and to nucleic acids and transformation vectors used.

Present systems for expressing PA for vaccine systems use protease deficient *Bacillus subtilis* as the expression host. Although such systems are acceptable in terms of product quantity and purity, there are significant drawbacks. Firstly, regulatory authorities are generally unfamiliar with this host, and licensing decisions may be delayed as a result. More importantly, the currently used strains of *Bacillus subtilis* produce thermostable spores which require the use of a dedicated production plant.

WO00/02522 describes in particular VEE virus replicons which express PA or certain immunogenic fragments.

E. coli is well known as an expression system for a range of human vaccines. While the ability to readily ferment *E. coli* to very high cellular densities makes this bacterium an ideal host for the expression of many proteins, previous attempts to express and purify recombinant PA from E. coli cytosol have been hindered by low protein yields and proteolytic degradation (Singh et al., J. Biol. Chem. (1989) 264; 11099-11102, Vodkin et al., Cell (1993) 34; 693-697 and Sharma et al., Protein Expr. purif. (1996), 7, 33-38).

A strategy for overexpressing PA as a stable, soluble protein in the *E. coli* cytosol has been described recently (Willhite et al., Protein and Peptide Letters, (1998), 5; 273-278). The strategy adopted is one of adding an affinity tag sequence to the N terminus of PA, which allows a simple purification system.

A problem with this system is that it requires a further downstream processing step in order to remove the tag before the PA can be used.

Codon optimisation is a technique which is now well known and used in the design of synthetic genes. There is a degree of redundancy in the genetic code, in so far as most amino acids are coded for by more than one codon sequence. Different organisms utilise one or other of these different codons preferentially. By optimising codons, it is generally expected that expression levels of the particular protein will be enhanced.

This is generally desirable, except where, as in the case of PA, higher expression levels will result in proteolytic degradation and/or cell toxicity. In such cases, elevating expression levels might be counter-productive and result in significant cell toxicity.

Surprisingly however, the applicants have found that this is not the case in *E. coli* and that in this system, codon optimisation results in expression of unexpectedly high levels of recombinant PA, irrespective of the presence or absence of proteolytic enzymes within the strain.

Furthermore, it would appear that expression of a protective domain of PA does not inhibit expression in *E. coli*.

The crystal structure of native PA has been elucidated (Petosa C., et al. Nature 385: 833-838,1997) and shows that PA consists of four distinct and functionally independent domains: domain 1, divided into 1a, 1-167 amino acids and 1b, 168-258 amino acids; domain 2, 259-487 amino acids; domain 3, 488-595 amino acids and domain 4, 596-735 amino acids.

The applicants have identified that certain domains appear to produce surprisingly good protective effects when used in isolation, in fusion proteins or in combination with each other.

According to the present invention there is provided an immunogenic reagent which produces an immune response which is protective against *Bacillus anthracis*, said reagent comprising one or more polypeptides which together represent up to three domains of the full length Protective Antigen (PA) of *B. anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof.

Specifically, the reagent will comprise mixtures of polypeptides or fusion peptides wherein individual polypeptides comprise one of more individual domains of PA.

In particular, the reagent comprises polypeptide(s) comprising domain 1 or domain 4 of PA or a variant thereof, in a form other than full length PA. Where present, domains are suitably complete, in particular domain 1 is present in its entirety.

The term "polypeptide" used herein includes proteins and peptides.

As used herein, the expression "variant" refers to sequences of amino acids which differ from the basic sequence in that one or more amino acids within the sequence are deleted or substituted for other amino acids, but which still produce an immune response which is protective against *Bacillus anthracis*. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% identical,

preferably at least 75% identical, and more preferably at least 90% identical to the PA sequence.

In particular, the identity of a particular variant sequence to the PA sequence may be assessed using the multiple alignment method described by Lipman and Pearson, (Lipman, D.J. & Pearson, W.R. (1985) Rapid and Sensitive Protein Similarity Searches, Science, vol 227, pp1435-1441). The "optimised" percentage score should be calculated with the following parameters for the Lipman-Pearson algorithm:ktup =1, gap penalty =4 and gap penalty length =12. The sequences for which similarity is to be assessed should be used as the "test sequence" which means that the base sequence for the comparison, (SEQ ID NO 1), should be entered first into the algorithm.

Preferably, the reagentof the invention includes a polypeptide which has the sequence of domain 1 and/or domain 4 of wild-type PA.

A particularly preferred embodiment of the invention comprises domain 4 of the PA of *B. anthracis*.

These domains comprise the following sequences shown in the following Table 1.

Table 1

Domain	Amino acids of full-length PA*
4	596-735
1	1-258

These amino acids numbers refer to the sequence as shown in Welkos et al. Gene 69 (1988) 287-300 and are illustrated hereinafter as SEQ ID NOs 15 (Fig 4) and 3 (Fig 3) respectively.

Domain 1 comprises two regions, designated 1a and 1b. Region 1a comprises amino acids 1-167 whereas region 1b is from amino acid 168-258. It appears that region 1a is important for the

production of a good protective response, and the full domain may be preferred.

In a particularly preferred embodiment, a combination of domains 1 and 4 or protective regions thereof, are used as the immunogenic reagent which gives rise to an immune response protective against *B. anthracis*. This combination, for example as a fusion peptide, may be expressed using the expression system of the invention as outlined hereinafter.

When domain 1 is employed, it is suitably fused to domain 2 of the PA sequence, and may preferably be fused to domain 2 and domain 3.

Such combinations and their use in prophylaxis or therapy forms a further aspect of the invention.

Suitably the domains described above are part of a fusion protein, preferably with an N-terminal glutathione-s-transferase protein (GST). The GST not only assists in the purification of the protein, it may also provide an adjuvant effect, possibly as a result of increasing the size.

The polypeptides of the invention are suitably prepared by conventional methods. For example, they may be synthesised or they may be prepared using recombinant DNA technology. In particular, nucleic acids which encode said domains are included in an expression vector, which is used to transform a host cell. Culture of the host cell followed by isolation of the desired polypeptide can then be carried out using conventional methods. Nucleic acids, vectors and transformed cells used in these methods form a further aspect of the invention.

Generally speaking, the host cells used will be those that are conventionally used in the preparation of PA, such as *Bacillus subtilis*.

The applicants have found surprisingly that the domains either in isolation or in combination, maybe successfully expressed in *E. coli* under certain conditions.

Thus, the present invention further provides a method for producing an immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising transforming an *E. coli* host with a nucleic acid which encodes either (a) the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, or (b) a polypeptide comprising at least one protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response as described above, culturing the transformed host and recovering the polypeptide therefrom, provided that where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 35%.

Using these options, high yields of product can be obtained using a favoured expression host.

A table showing codons and the frequency with which they appear in the genomes of Escherichia coli and *Bacillus anthracis* is shown in Figure 1. It is clear that guanidine and cytosine appear much more frequently in *E.coli* than *B. anthracis*. Analysis of the codon usage content reveals the following:

Species	1st letter of Codon GC	2nd letter of Codon GC	3rd letter of Codon GC	Total GC content
E. coli	58.50%	40.70%	54.90%	51.37%
B. anthracis	44.51%	31.07%	25.20%	33.59%

Thus it would appear that codons which are favoured by *E. coli* are those which include guanidine or cytosine where possible.

By increasing the percentage of guanidine and cytosine nucleotides in the sequence used to encode the immunogenic protein over that normally found in the wild-type *B. anthracis* gene, the codon usage will be such that expression in *E. coli* is improved.

Suitably the percentage of guanidine and cytosine residues within the coding nucleic acid used in the invention, at least where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, is in excess of 40%, preferably in excess of 45% and most preferably from 50-52%.

High levels of expression of protective domains can be achieved, with using the wild-type *B. anthracis* sequence encoding these units. However, the yields may be improved further by increasing the GC% of the nucleic acid as described above.

In a particular embodiment, the method involves the expression of PA of *B. anthracis*.

Further according to the present invention, there is provided a recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

As before, suitably the percentage of guanidine and cytosine residues within the coding nucleic acid is in excess of 40%, preferably in excess of 45% and most preferably from 50-52%.

Suitably, the nucleic acid used to transform the *E. coli* cells of the invention is a synthetic gene. In particular, the

nucleic acid is of SEQ ID NO 1 as shown in Figure 2 or a modified form thereof.

The expression "modified form" refers to other nucleic acid sequences which encode PA or fragments or variants thereof which produce a protective immune response but which utilise some different codons, provided the requirement for the percentage GC content in accordance with the invention is met. Suitable modified forms will be at least 80% similar, preferably 90% similar and most preferably at least 95% similar to SEQ ID NO 1. In particular, the nucleic acid comprises SEQ ID NO 1.

In an alternative embodiment, the invention provides a recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

Preferably, the nucleic acid encodes domain 1 or domain 4 of *B. anthracis*.

Further according to the invention there is provided a method of producing immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising culturing a cell as described above and recovering the desired polypeptide from the culture. Such methods are well known in the art.

In yet a further aspect, the invention provides an *E. coli* transformation vector comprising a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

A still further aspect of the invention comprises an *E. coli* transformation vector comprising a nucleic acid which encodes a protective domain *of* the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

Suitable vectors for use in the transformation of *E. coli* are well known in the art. For example, the T7 expression system provides good expression levels. However a particularly preferred vector comprises pAG163 obtainable from Avecia (UK).

A nucleic acid of SEQ ID NO 1 or a variant thereof which encodes PA and which has at 35%, preferably at least 40%, more preferably at least 45% and most preferably from 50-52% GC content form a further aspect of the invention.

If desired, PA of the variants, or domains can be expressed as a fusion to another protein, for example a protein which provides a different immunity, a protein which will assist in purification of the product or a highly expressed protein (e.g. thioredoxin, GST) to ensure good initiation of translation.

Optionally, additional systems will be added such as T7 lysozyme to the expression system, to improve the repression of the system, although, in the case of the invention, the problems associated with cell toxicity have not been noted.

Any suitable *E. coli* strain can be employed in the process of the invention. Strains which are deficient in a number of proteases (e.g. Ion^-, $ompT^-$) are available, which would be expected to minimise proteolysis. However, the applicants have found that there is no need to use such strains to achieve good yields of product and that other known strains such as K12 produce surprisingly high product yields.

Fermentation of the strain is generally carried out under conventional conditions as would be understood in the art. For example, fermentations can be carried out as batch cultures, preferably in large shake flasks, using a complex medium containing antibiotics for plasmid maintenance and with addition of IPTG for induction.

Suitably cultures are harvested and cells stored at -20°C until required for purification.

Suitable purification schemes for *E. coli* PA (or variant or domain) expression can be adapted from those used in *B. subtilis* expression. The individual purification steps to be used will depend on the physical characteristics of recombinant PA. Typically an ion exchange chromatography separation is carried out under conditions which allow greatest differential binding to the column followed by collection of fractions from a shallow gradient. In some cases, a single chromatographic step may be sufficient to obtain product of the desired specification.

Fractions can be analysed for the presence of the product using SDS PAGE or Western blotting as required.

As illustrated hereinafter, the successful cloning and expression of a panel of fusion proteins representing intact or partial domains of rPA has been achieved. The immunogenicity and protective efficacy of these fusion proteins against STI spore challenge has been assessed in the A/J mouse model.

All the rPA domain proteins were immunogenic in A/J mice and conferred at least partial protection against challenge compared to the GST control immunised mice. The carrier protein, GST attached to the N-terminus of the domain proteins, did not impair the immunogenicity of the fusion proteins either in vivo, shown by the antibody response stimulated in immunised animals, or in vitro as the fusion proteins could be detected with anti-

rPA antisera after Western blotting, indicating that the GST tag did not interfere with rPA epitope recognition. Immunisation with the larger fusion proteins produced the highest titres. In particular, mice immunised with the full length GST 1-4 fusion protein produced a mean serum anti-rPA concentration approximately eight times that of the rPA immunised group (Figure 5). Immunisation of mice with rPA domains 1-4 with the GST cleaved off, produced titres of approximately one half those produced by immunisation with the fusion protein. Why this fusion protein should be much more immunogenic is unclear. It is possible that the increased size of this protein may have an adjuvantising effect on the immune effector cells. It did not stimulate this response to the same extent in the other fusion proteins and any adjuvantising effect of the GST tag did not enhance protection against challenge as the cleaved proteins were similarly protective to their fusion protein counterparts.

Despite having good anti-rPA titres, some breakthrough in protection at the lower challenge level of 10^2MLD's, occurred in the groups immunised with GST1, cleaved 1, GST1b-2, GST1b-3 and GST1-3 and immunisation with these proteins did not prolong the survival time of those mice that did succumb to challenge, compared with the GST control immunised mice. This suggests that the immune response had not been appropriately primed by these proteins to achieve full resistance to the infection. As has been shown in other studies in mice and guinea pigs (Little S.F. et al. 1986. Infect. Immun. 52: 509-512, Turnbull P.C.B., et al., 1986. Infect. Immun. 52: 356-363) there is no precise correlation between antibody titre to PA and protection against challenge. However a certain threshold of antibody is required for protection (Cohen S et. al., 2000 Infect. Immun. 68: 4549-4558), suggesting that cell mediated components of the immune response are also required to be stimulated for protection (Williamson 1989).

GST1, GST1b-2 and GST1-2 were the least stable fusion proteins produced, as shown by SDS-Page and Western blotting results, possibly due to the proteins being more susceptible to degradation in the absence of domain 3, and this instability may have resulted in the loss of protective epitopes.

The structural conformation of the proteins may also be important for stimulating a protective immune response. The removal of Domain 1a from the fusion proteins gave both reduced antibody titres and less protection against challenge, when compared to their intact counterparts GST1-2 and GST1-3. Similarly, mice immunised with GST 1 alone were partially protected against challenge, but when combined with domain 2, as the GST1-2 fusion protein, full protection was seen at the 10^2 MLD challenge level. However the immune response stimulated by immunisation with the GST1-2 fusion protein was insufficient to provide full protection against the higher 10^3 MLD's challenge level, which again could be due to the loss of protective epitopes due to degradation of the protein.

All groups immunised with truncates containing domain 4, including GST 4 alone, cleaved 4 alone and a mixture of two individually expressed domains, GST 1 and GST 4 were fully protected against challenge with 10^3 MLDs of STI spores (Table 1). Brossier *et al* showed a decrease in protection in mice immunised with a mutated strain of *B.anthracis* that expressed PA without domain 4 (Brossier F., et al. 2000. Infect. Immun. 68: 1781-1786) and this was confirmed in this study, where immunisation with GST 1-3 resulted in breakthrough in protection despite good antibody titres. These data indicate that domain 4 is the immunodominant sub-unit of PA. Domain 4 represents the 139 amino acids of the carboxy terminus of the PA polypeptide. It contains the host cell receptor binding region (Little S.F. et al., 1996 Microbiology 142: 707-715), identified as being in and near a small loop located between amino acid residues 679-693 (Varughese M., et al. 1999 Infect. Immun. 67:1860-1865).

Therefore it is essential for host cell intoxication as it has been demonstrated that forms of PA expressed containing mutations (Varughese 1999 supra.) or deletions (Brossier 1999 supra.) in the region of domain 4 are non-toxic. The crystal structure of PA shows domain 4, and in particular a 19 amino acid loop of the domain (703-722), to be more exposed than the other three domains which are closely associated with each other (Petosa 1997 supra.). This structural arrangement may make domain 4 the most prominent epitope for recognition by immune effector cells, and therefore fusion proteins containing domain 4 would elicit the most protective immune response.

This investigation has further elucidated the role of PA in the stimulation of a protective immune response demonstrating that protection against anthrax infection can be attributed to individual domains of PA.

The invention will now be particularly described by way of example, with reference to the accompanying drawings in which:

Figure 1 is a Table of codon frequencies found within *E. coli* and *B. anthracis*;

Figure 2 shows the sequence of a nucleic acid according to the invention, which encodes PA of B. subtilis, as published by Welkos et al supra; and

Figure 3 shows SEQ ID NOs 3-14, which are amino acid and DNA sequences used to encode various domains or combinations of domains of PA as detailed hereinafter;

Figure 4 shows SEQ ID NOs 15-16 which are the amino acid and DNA sequences of domain 4 of PA respectively; and

Figure 5 is a table showing anti-rPA IgG concencentration, 37 days post primary immunisation, from A/J mice immunised

intramuscularly on days 1 and 28 with 10µg of fusion protein included PA fragment; results shown are mean ± sem of samples taken from 5 mice per treatment group.

Example 1

Investigation into expression in E.coli

rPA expression plasmid pAG163::rPA has been modified to substitute Km^R marker for original Tc^R gene. This plasmid has been transformed into expression host *E. coli* BLR (DE3) and expression level and solubility assessed. This strain is deficient in the intracellular protease La (Ion gene product) and the outer membrane protease OmpT.

Expression studies did not however show any improvement in the accumulation of soluble protein in this strain compared to Ion+ K12 host strains (i.e. accumulation is prevented due to excessive proteolysis). It was concluded that any intracellular proteolysis of rPA was not due to the action of La protease.

Example 2

Fermentation analysis

Further analysis of the fermentation that was done using the K12 strain UT5600 (DE3) pAG163::rPA.

It was found that the rPA in this culture was divided between the soluble and insoluble fractions (estimated 350mg/L insoluble, 650mg/L full length soluble). The conditions used (37°C, 1mM IPTG for induction) had not yielded any detectable soluble rPA in shake flask cultures and given the results described in Example 1 above, the presence of a large amount of soluble rPA is surprising. Nevertheless it appears that manipulation of the fermentation, induction and point of harvest may allow stable accumulation of rPA in *E. coli* K12 expression strains.

Example 3

A sample of rPA was produced from material initially isolated as insoluble inclusion bodies from the UT5600 (DE3) pAG163::rPA fermentation. Inclusion bodies were washed twice with 25mM Tris-HCl pH8 and once with same buffer +2M urea. They were then solubilized in buffer +8M urea and debris pelleted. Urea was removed by dilution into 25mM Tris-HCl pH8 and static incubation overnight at 4°C. Diluted sample was applied to Q sepharose column and protein eluted with NaCl gradient. Fractions containing highest purity rPA were pooled, aliquoted and frozen at -70°C. Testing of this sample using 4-12% MES-SDS NuPAGE gel against a known standard indicated that it is high purity and low in endotoxin contamination.

Example 4

Further Characterisation of the Product

N terminal sequencing of the product showed that the N-terminal sequence consisted of

MEVKQENRLL (SEQ ID NO 2)

This confirmed that the product was as expected with initiator methionine left on.

The material was found to react in Western blot; MALDI -MS on the sample indicated a mass of approx 82 700 (compared to expected mass of 82 915). Given the high molecular mass and distance from mass standard used (66KDa), this is considered an indication that material does not have significant truncation but does not rule out microheterogeneity within the sample.

Example 5

Testing of Individual domains of PA

Individual domains of PA were produced as recombinant proteins in *E.coli* as fusion proteins with the carrier protein glutathione-*s*-transferase (GST), using the Pharmacia pGEX-6P-3 expression system. The sequences of the various domains and

the DNA sequence used to encode them are attached herewith as Figure 3. The respective amino acid and DNA sequences are provided in Table 2 below.

These fusion proteins were used to immunise A/J mice (Harlan Olac) intra-muscularly with 10μg of the respective fusion protein adsorbed to 20% v/v alhydrogel in a total volume of 100μl.

Animals were immunised on two occasions and their development of protective immunity was determined by challenge with spores of B.anthracis (STI strain) at the indicated dose levels. The table below shows survivors at 14 days post-challenge.

Challenge level in spores/mouse

Domains	Amino acid SEQ ID NO	DNA SEQ ID NO	$5x10^4$	$9x10^4$	$9x10^5$	$1x10^6$	$5x10^6$
GST-1	3	4	4/4	3/5			
GST-1+2	5	6	4/4; 5/5	4/5; 5/5			
GST-1b+2	7	8	2/5	1/5			
GST-1b+2+3	9	10	2/5	3/5			
GST-1+2+3	11	12	Nd	4/5	3/5		
GST-1+2+3+4	13	14	Nd	5/5	5/5		
1+2+3+4	13	14	Nd	Nd		5/5	5/5

The data shows that a combination of all 4 domains of PA, whether presented as a fusion protein with GST or not, were protective up to a high challenge level. Removal of domain 4, leaving 1+2+3, resulted in breakthrough at the highest challenge level tested, $9x10^5$. Domains 1+2 were as protective as a combination of domains 1+2+3 at $9x10^4$ spores. However, removal of domain 1a to leave a GST fusion with domains 1b+2, resulted in breakthrough in protection at the highest challenge level

tested ($9x10^{4}$) which was only slightly improved by adding domain 3.

The data indicates that the protective immunity induced by PA can be attributed to individual domains (intact domain 1 and domain 4) or to combinations of domains taken as permutations from all 4 domains.

The amino acid sequence and a DNA coding sequence for domain 4 is shown in Figure 4 as SEQ ID NOs 15 and 16 respectively.

Example 6

Further Testing of domains as vaccines

DNA encoding the PA domains, amino acids 1-259, 168-488, 1-488, 168-596,1-596, 260-735, 489-735, 597-735 and 1-735 (truncates GST1, GST1b-2, GST1-2, GST1b-3, GST1-3, GST2-4, GST3-4, GST4 and GST1-4 respectively) were PCR amplified from *B. anthracis* Sterne DNA and cloned in to the *XhoI/BamHI* sites of the expression vector pGEX-6-P3 (Amersham-Pharmacia) downstream and in frame of the *lac* promoter. Proteins produced using this system were expressed as fusion proteins with an N-terminal glutathione-s-transferase protein (GST). Recombinant plasmid DNA harbouring the DNA encoding the PA domains was then transformed in to *E. coli* BL21 for protein expression studies.

E.coli BL21 harbouring recombinant pGEX-6-P3 plasmids were cultured in L-broth containing 50µg/ml ampicillin, 30µg/ml chloramphenicol and 1% w/v glucose. Cultures were incubated with shaking (170 rev min^{-1}) at 30°C to an A_{600nm} 0.4, prior to induction with 0.5mM IPTG. Cultures were incubated for a further 4 hours, followed by harvesting by centrifugation at 10 000 rpm for 15 minutes.

Initial extraction of the PA truncates-fusion proteins indicated that they were produced as inclusion bodies. Cell pellets were resuspended in phosphate buffered saline (PBS) and sonicated

4x20 seconds in an iced water bath. The suspension was centrifuged at 15 000 rpm for 15 minutes and cell pellets were then urea extracted, by suspension in 8M urea with stirring at room temperature for 1 hour. The suspension was centrifuged for 15 minutes at 15000 rpm and the supernatant dialysed against 100mM Tris pH 8 containing 400mM L-arginine and 0.1mM EDTA, prior to dialysis into PBS.

The successful refolding of the PA truncate-fusion proteins allowed them to be purified on a glutathione Sepharose CL-4B affinity column. All extracts (with the exception of truncate GST1b-2, amino acid residues 168-487) were applied to a 15 ml glutathione Sepharose CL-4B column (Amersham-Parmacia), previously equilibrated with PBS and incubated, with rolling, overnight at 4°C. The column was washed with PBS and the fusion protein eluted with 50mM Tris pH7, containing 150mM NaCl, 1mM EDTA and 20mM reduced glutathione. Fractions containing the PA truncates, identified by SDS-PAGE analysis, were pooled and dialysed against PBS. Protein concentration was determined using BCA (Perbio).

However truncate GST1b-2 could not be eluted from the glutathione sepharose CL-4B affinity column using reduced glutathione and was therefore purified using ion exchange chromatography. Specifically, truncate GST1b-2 was dialysed against 20mM Tris pH8, prior to loading onto a HiTrap Q column (Amersham-Parmacia), equilibrated with the same buffer. Fusion protein was eluted with an increasing NaCl gradient of 0-1M in 20mM Tris pH8. Fractions containing the GST-protein were pooled, concentrated and loaded onto a HiLoad 26/60 Superdex 200 gel filtration column (Amersham-Parmacia), previously equilibrated with PBS. Fractions containing fusion protein were pooled and the protein concentration determined by BCA (Perbio). Yields were between 1 and 43mg per litre of culture.

The molecular weight of the fragments and their recognition by antibodies to PA was confirmed using SDS PAGE and Western Blotting. Analysis of the rPA truncates by SDS Page and Western blotting showed protein bands of the expected sizes. Some degradation in all of the rPA truncates investigated was apparent showing similarity with recombinant PA expressed in *B. subtilis*. The rPA truncates GST1, GST1b-2 and GST1-2 were particularly susceptible to degradation in the absence of domain 3. This has similarly been reported for rPA constructs containing mutations in domain 3, that could not be purified from *B. anthracis* culture supernatants (Brossier 1999), indicating that domain 3 may stabilise domains 1 and 2.

Female, specific pathogen free A/J mice (Harlan UK) were used in this study as these are a consistent model for anthrax infection (Welkos 1986). Mice were age matched and seven weeks of age at the start of the study.

A/J mice were immunised on days 1 and 28 of the study with 10µg of fusion protein adsorbed to 20% of 1.3% v/v Alhydrogel (HCI Biosector, Denmark) in a total volume of 100µl of PBS. Groups immunised with rPA from *B.subtilis* (Miller 1998), with recombinant GST control protein, or fusion proteins encoding domains 1, 4 and 1-4 which had the GST tag removed, were also included. Immunising doses were administered intramuscularly into two sites on the hind legs. Mice were blood sampled 37 days post primary immunisation for serum antibody analysis by enzyme linked immunosorbant assay (ELISA).

Microtitre plates (Immulon 2, Dynex Technologies) were coated, overnight at 4° C with 5µg/ml rPA, expressed from *B.subtilis* (Miller1998), in PBS except for two rows per plate which were coated with 5µg/ml anti-mouse Fab (Sigma, Poole, Dorset). Plates were washed with PBS containing 1% v/v Tween 20 (PBS-T) and blocked with 5% w/v skimmed milk powder in PBS (blotto) for 2 hours at 37° C. Serum, double-diluted in 1% blotto, was added

to the rPA coated wells and was assayed in duplicate together with murine IgG standard (Sigma) added to the anti-fab coated wells and incubated overnight at 4^0 C. After washing, horse-radish peroxidase conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc.), diluted 1 in 2000 in PBS, was added to all wells, and incubated for 1 hour at 37^0 C. Plates were washed again before addition of the substrate 2,2'-Azinobis (3-ethylbenzthiazoline-sulfonic acid) (1.09mM ABTS, Sigma). After 20 minutes incubation at room temperature, the absorbance of the wells at 414nm was measured (Titertek Multiscan, ICN Flow). Standard curves were calculated using Titersoft version 3.1c software. Titres were presented as µg IgG per ml serum and group means ± standard error of the mean (sem) were calculated. The results are shown in Figure 5.

All the rPA truncates produced were immunogenic and stimulated mean serum anti-rPA IgG concentrations in the A/J mice ranging from 6µg per ml, for the GST1b-2 truncate immunised group, to 1488µg per ml, in the GST 1-4 truncate immunised group (Figure 5). The GST control immunised mice had no detectable antibodies to rPA.

Mice were challenged with B.anthracis STI spores on day 70 of the immunisation regimen. Sufficient STI spores for the challenge were removed from stock, washed in sterile distilled water and resuspended in PBS to a concentration of $1x10^7$ and $1x10^6$ spores per ml. Mice were challenged intraperitoneally with 0.1ml volumes containing $1x10^6$ and $1x10^5$ spores per mouse, respectively, and were monitored for 14 day post challenge to determine their protected status. Humane end-points were strictly observed so that any animal displaying a collection of clinical signs which together indicated it had a lethal infection, was culled. The numbers of immunised mice which survived 14 days post challenge are shown in Table 3.

Table 3

Domain	Challenge Level MLDs survivors/no. challenged (%)	
	10^2 MLDs	10^3 MLDs
GST 1	3/5 (60)	1/5 (20)
GST 1b-2	1/5 (20)	nd
GST 1-2	5/5 (100)	3/5 (60)
GST 1b-3	3/5 (60)	nd
GST 1-3	4/5 (80)	nd
GST 1-4	nd	5/5 (100)
GST 2-4	nd	5/5 (100)
GST 3-4	nd	5/5 (100)
GST 4	5/5 (100)	5/5 (100)
GST 1+ GST 4	nd	5/5 (100)
Cleaved 1	1/5 (20)	2/5
Cleaved 4	5/5 (100)	5/5
Cleaved 1-4	nd	5/5
rPA	nd	4/4 (100)
control	0/5 (0)	0/5 (0)

1 MLD = aprox. 1 x 10^3 STI spores
nd = not done

The groups challenged with 10^3 MLD's of STI spores were all fully protected except for the GST1, GST1-2 and cleaved 1 immunised groups in which there was some breakthrough in protection, and the control group immunised with GST only, which all succumbed to infection with a mean time to death (MTTD) of 2.4 $\pm$ 0.2 days. At the lower challenge level of 10^2 MLD's the GST1-2, GST4 and cleaved 4 - immunised groups were all fully protected, but there was some breakthrough in protection in the other groups. The mice that died in these groups had a MTTD of 4.5 $\pm$ 0.2 days which was not significantly different from the GST control immunised group which all died with a MTTD of 4 $\pm$ 0.4 days.

Claims

1. An immunogenic reagent which produces an immune response which is protective against *Bacillus anthracis*, said reagent comprising one or more polypeptides which together represent up to three domains of the full length Protective Antigen (PA) of *B. anthracis* or variants of these, and at least one of said domains comprises domain 1 or domain 4 of PA or a variant thereof.

2. An immunogenic reagent according to claim 1 which comprises the sequence of domain 1 and/or domain 4 of wild-type PA.

3. An immunogenic reagent according to claim 1 or claim 2 which comprises domain 4 of the PA of *B. anthracis*.

4. An immunogenic reagent according to any one of the preceding claims which comprises a combination of domains 1 and 4 or protective regions thereof.

5. An immunogenic reagent according to claim 4 wherein said domains are present in the form of a fusion polypeptide.

6. An immunogenic reagent according to claim 5 which comprises domain 1 fused to domain 2 of the PA sequence.

7. An immunogenic reagent according to claim 6 which is fused to domain 3 of the PA sequence.

8. An immunogenic reagent according to claim 4 which comprises a mixture of a polypeptides, one of which comprises domain 1 and one of which comprises domain 4 of the PA sequence.

9. An immunogenic reagent according to any one of the preceding claims wherein a polypeptide is fused to a further polypeptide.

10. An immunogenic reagent according to claim 9 wherein said further peptide is glutathione-S-transferase (GST).

11. A nucleic acid which encodes a polypeptide of an immunogenic reagent according to any one of the preceding claims.

12. An expression vector comprising a nucleic acid according to claim 11.

13. A cell transformed with a vector according to claim 12.

14. A method for producing an immunogenic polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising transforming an *E. coli* host with a nucleic acid which encodes either (a) the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, or (b) a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, culturing the transformed host and recovering the polypeptide therefrom, provided that where the polypeptide is the protective antigen (PA) of *Bacillus anthracis* a variant thereof which can produce a protective immune response, the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 35%.

15. A method according to claim 14 wherein the said nucleic acid encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

16. A method according to claim 15 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 45%.

17. A method according to claim 16 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is from 50-52%.

18. A method according to claim 14 wherein the said nucleic acid encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

19. A method according to claim 18 wherein the domain is domain 1 and/or domain 4 of PA of *B. anthracis*.

20. A recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

21. A recombinant *Escherischia coli* cell according to claim 20 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is in excess of 45%.

22. A recombinant *Escherischia coli* cell according to claim 21 wherein the percentage of guanidine and cytosine residues within the said nucleic acid is from 50%-52%.

23. A recombinant *E. coli* cell according to claim 20 wherein said nucleic acid is of SEQ ID NO 1 as shown in Figure 2 or a modified form thereof.

24. A recombinant *E. coli* cell according to claim 23 wherein said nucleic acid is of SEQ ID NO 1.

25. A recombinant *Escherischia coli* cell which has been transformed with a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

26. A recombinant cell according to claim 25 wherein the nucleic acid encodes domain 1 or domain 4 of PA of *B. anthracis*.

27. A method of producing a polypeptide which produces an immune response which is protective against *B. anthracis*, said method comprising culturing a cell according to any one of claims 20 to 26 and recovering the protective polypeptide from the culture.

28. An *E. coli* transformation vector comprising a nucleic acid which encodes the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response, and wherein the percentage of guanidine and cytosine residues within the nucleic acid is in excess of 35%.

29. An *E. coli* transformation vector comprising a nucleic acid which encodes a protective domain of the protective antigen (PA) of *Bacillus anthracis* or a variant thereof which can produce a protective immune response.

30. A nucleic acid of SEQ ID NO 1 or a modified form thereof which encodes PA or a variant thereof which produces a protective immune response and which has at least 35% GC content.

31. A nucleic acid according to claim 30 which is at least 90% identical to SEQ ID NO 1.

32. A nucleic acid according to claim 31 which comprises SEQ ID NO 1.

34. A method of preventing or treating infection by B. anthracis, said method comprising administering to a mammal in need thereof, a sufficient amount of an immunogenic reagent according to any one of claims 1 to 10.

35. The use of an immunogenic reagent according to any one of claims 1 to 10 in the preparation of a medicament for the prophylaxis or treatment of *B. anthracis* infection.

Escherichia coli [gbbct]: 14457 CDS's (4541860 codons)

Fields: [triplet] [frequency: per thousand] ([number])

UUU 22.0(100126)	UCU 9.3(42367)	UAU 16.7(75774)	UGU 5.2(23461)
UUC 16.5(74885)	UCC 8.9(40365)	UAC 12.3(55847)	UGC 6.3(28747)
UUA 13.8(62823)	UCA 7.9(35837)	UAA 2.0(9006)	UGA 1.0(4428)
UUG 13.3(60322)	UCG 8.7(39546)	UAG 0.3(1172)	UGG 14.5(65630)
CUU 11.3(51442)	CCU 7.2(32678)	CAU 12.7(57585)	CGU 20.7(93997)
CUC 10.6(48147)	CCC 5.4(24383)	CAC 9.6(43743)	CGC 21.1(96053)
CUA 4.0(18067)	CCA 8.5(38663)	CAA 14.8(67129)	CGA 3.7(16607)
CUG 50.9(231373)	CCG 22.3(101467)	CAG 28.8(130898)	CGG 5.7(25751)
AUU 29.9(135873)	ACU 9.5(43256)	AAU 18.7(84846)	AGU 9.1(41544)
AUC 24.6(111878)	ACC 22.7(103121)	AAC 21.6(98018)	AGC 15.6(70867)
AUA 5.3(24233)	ACA 7.9(35995)	AAA 34.4(156169)	AGA 2.7(12345)
AUG 27.2(123604)	ACG 14.0(63696)	AAG 11.4(51685)	AGG 1.6(7423)
GUU 19.1(86572)	GCU 16.2(73677)	GAU 32.3(146794)	GGU 25.1(114185)
GUC 14.8(67356)	GCC 25.0(113412)	GAC 19.3(87759)	GGC 28.6(130043)
GUA 11.2(51020)	GCA 20.6(93390)	GAA 39.5(179460)	GGA 8.6(39036)
GUG 25.5(115687)	GCG 32.2(146264)	GAG 18.5(83804)	GGG 11.1(50527)

Coding GC 51.37% 1st letter GC 58.50% 2nd letter GC 40.70% 3rd letter GC 54.90%

Bacillus anthracis [gbbct]: 180 CDS's (52031 codons)

Fields: [triplet] [frequency: per thousand] ([number])

UUU 33.5(1745)	UCU 17.3(902)	UAU 34.4(1792)	UGU 6.1(319)
UUC 10.2(530)	UCC 5.3(275)	UAC 9.4(490)	UGC 2.1(107)
UUA 44.2(2301)	UCA 14.0(730)	UAA 2.3(118)	UGA 0.5(24)
UUG 11.3(589)	UCG 3.6(188)	UAG 0.7(37)	UGG 9.8(511)
CUU 14.7(763)	CCU 10.1(525)	CAU 16.8(873)	CGU 10.9(567)
CUC 3.7(195)	CCC 2.7(141)	CAC 4.6(239)	CGC 2.6(137)
CUA 13.2(686)	CCA 14.9(773)	CAA 33.7(1752)	CGA 6.8(353)
CUG 4.7(242)	CCG 4.6(237)	CAG 10.4(542)	CGG 1.8(95)
AUU 44.6(2322)	ACU 14.6(761)	AAU 44.6(2321)	AGU 16.5(861)
AUC 11.8(616)	ACC 5.2(269)	AAC 13.7(711)	AGC 5.1(266)
AUA 24.9(1295)	ACA 25.9(1350)	AAA 69.5(3614)	AGA 13.8(720)
AUG 23.8(1240)	ACG 8.1(419)	AAG 23.5(1223)	AGG 4.3(226)
GUU 19.9(1036)	GCU 17.9(930)	GAU 39.7(2068)	GGU 17.3(900)
GUC 5.2(268)	GCC 4.7(244)	GAC 8.8(456)	GGC 5.4(279)
GUA 26.8(1395)	GCA 22.6(1178)	GAA 55.7(2897)	GGA 20.2(1049)
GUG 9.7(507)	GCG 7.1(368)	GAG 19.3(1003)	GGG 8.9(461)

Coding GC 33.59% 1st letter GC 44.51% 2nd letter GC 31.07% 3rd letter GC 25.20%

Figure 1

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   1 AAGCTTCATA TGGAAGTAAA GCAAGAGAAC CGTCTGCTGA ACGAATCTGA ATCCAGCTCT
  61 CAGGGCCTGC TTGGTTACTA TTTCTCTGAC CTGAACTTCC AAGCACCGAT GGTTGTAACC
 121 AGCTCTACCA CTGGCGATCT GTCCATCCCG TCTAGTGAAC TTGAGAACAT TCCAAGCGAG
 181 AACCAGTATT TCCAGTCTGC AATCTGGTCC GGTTTTATCA AAGTCAAGAA ATCTGATGAA
 241 TACACGTTTG CCACCTCTGC TGATAACCAC GTAACCATGT GGGTTGACGA TCAGGAAGTG
 301 ATCAACAAAG CATCCAACTC CAACAAAATT CGTCTGGAAA AAGGCCGTCT GTATCAGATC
 361 AAGATTCAGT ACCAACGCGA GAACCCGACT GAAAAAGGCC TGGACTTTAA ACTGTATTGG
 421 ACTGATTCTC AGAACAAGAA AGAAGTGATC AGCTCTGACA ATCTGCAACT GCCGGAATTG
 481 AAACAGAAAA GCTCCAACTC TCGTAAGAAA CGTTCCACCA GCGCTGGCCC GACCGTACCA
 541 GATCGCGACA ACGATGGTAT TCCGGACTCT CTGGAAGTTG AAGGCTACAC GGTTGATGTA
 601 AAGAACAAAC GTACCTTCCT TAGTCCGTGG ATCTCCAATA TTCACGAGAA GAAAGGTCTG
 661 ACCAAATACA AATCCAGTCC GGAAAAATGG TCCACTGCAT CTGATCCGTA CTCTGACTTT
 721 GAGAAAGTGA CCGGTCGTAT CGACAAGAAC GTCTCTCCGG AAGCACGCCA TCCACTGGTT
 781 GCTGCGTATC CGATCGTACA TGTTGACATG GAAAACATCA TTTTGTCCAA GAACGAAGAC
 841 CAGTCCACTC AGAACACTGA CTCTGAAACT CGTACCATCT CCAAGAACAC CTCCACGTCT
 901 CGTACTCACA CCAGTGAAGT ACATGGTAAC GCTGAAGTAC ACGCCTCTTT CTTTGACATC
 961 GGCGGCTCTG TTAGCGCTGG CTTCTCCAAC TCTAATTCTT CTACTGTTGC CATTGATCAC
1021 TCTCTGAGTC TGGCTGGCGA ACGTACCTGG GCAGAGACCA TGGGTCTTAA CACTGCTGAT
1081 ACCGCGCGTC TGAATGCTAA CATTCGCTAC GTCAACACTG GTACGGCACC GATCTACAAC
1141 GTACTGCCAA CCACCAGCCT GGTTCTGGGT AAGAACCAGA CTCTTGCGAC CATCAAAGCC
1201 AAAGAGAACC AACTGTCTCA GATTCTGGCA CCGAATAACT ACTATCCTTC CAAGAACCTG
1261 GCTCCGATCG CACTGAACGC ACAGGATGAC TTCTCTTCCA CTCCGATCAC CATGAACTAC
1321 AACCAGTTCC TGGAACTTGA GAAGACCAAA CAGCTGCGTC TTGACACTGA CCAAGTGTAC
1381 GGTAACATCG CGACCTACAA CTTTGAGAAC GGTCGCGTCC GCGTTGACAC AGGCTCTAAT
1441 TGGTCTGAAG TACTGCCTCA GATTCAGGAA ACCACCGCTC GTATCATCTT CAACGGTAAA
1501 GACCTGAACC TGGTTGAACG TCGTATTGCT GCTGTGAACC CGTCTGATCC ATTAGAGACC
1561 ACCAAACCGG ATATGACTCT GAAAGAAGCC CTGAAGATCG CCTTTGGCTT CAACGAGCCG
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1681 CAGACCTCTC AGAATATCAA GAACCAACTG GCTGAGCTGA ACGCGACCAA TATCTATACG
1741 GTACTCGACA AGATCAAACT GAACGCGAAA ATGAACATTC TGATTCGCGA CAAACGTTTC
1801 CACTACGATC GTAATAACAT CGCTGTTGGC GCTGATGAAT CTGTTGTGAA AGAAGCGCAT
1861 CGCGAAGTCA TCAACTCCAG CACCGAAGGC CTGCTTCTGA ACATCGACAA AGACATTCGT
1921 AAGATCCTGT CTGGTTACAT TGTTGAGATC GAAGACACCG AAGGCCTGAA AGAAGTGATC
1981 AATGATCGTT ACGACATGCT GAACATCAGC TCTCTGCGTC AAGATGGTAA GACGTTCATT
2041 GACTTCAAGA AATACAACGA CAAACTTCCG CTGTATATCT CTAATCCGAA CTACAAAGTG
2101 AACGTTTACG CTGTTACCAA AGAGAACACC ATCATCAATC CATCTGAGAA CGGCGATACC
2161 TCTACCAACG GTATCAAGAA GATTCTGATC TTCTCCAAGA AAGGTTACGA GATCGGTTAA
2221 TAGGATCC
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(SEQ ID No 1)

Figure 2

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  1 EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL ENIPSENQYF
 61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAA
```

(Seq ID No 3)

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 61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt
181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat
361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
421 ataaaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct
481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
601 actttttctt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa
661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca
721 ggacggattg ataagaatgt atcaccagag gcaagacacc cccttgtggc agct
```

(Seq ID No 4)

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 61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRTHT
301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL
361 NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
481 LPQIQET
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(SEQ ID No 5)

Figure 3

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   1 gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta
  61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacttc ttctactaca
 121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt
 181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
 241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
 301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat
 361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
 421 aataaaaaag aagtgatttc tagtgataac ttacaactgc cagaattaaa acaaaaatct
 481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
 541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
 601 acttttcttt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa
 661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca
 721 ggacggattg ataagaatgt atcaccagag gcaagacacc ccttgtggc agcttatccg
 781 attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag
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 901 agtgaagtac atggaaatgc agaagtgcat gcgtcgttct ttgatattgg tgggagtgta
 961 tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta
1021 gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata ccgctgatac agcaagatta
1081 aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg
1141 acttcgttag tgttaggaaa aaatcaaaca ctcgcgacaa ttaaagctaa ggaaaaccaa
1201 ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca
1261 ttaaatgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt
1321 gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca
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1441 ttaccgcaaa ttcaagaaac a
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(SEQ ID No 6)

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   1 SAGPTVPDRD NDGIPDSLEV EGYTVDVKNK RTFLSPWISN IHEKKGLTKY KSSPEKWSTA
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 121 SKNTSTSRTH TSEVHGNAEV HASFFDIGGS VSAGFSNSNS STVAIDHSLS LAGERTWAET
 181 MGLNTADTAR LNANIRYVNT GTAPIYNVLP TTSLVLGKNQ TLATIKAKEN QLSQILAPNN
 241 YYPSKNLAPI ALNAQDDFSS TPITMNYNQF LELEKTKQLR LDTDQVYGNI ATYNFENGRV
 301 RVDTGSNWSE VLPQIQET
```

(SEQ ID No 7)

Figure 3 Cont.

```
   1 agtgctggac ctacggttcc agaccgtgac aatgatggaa tccctgattc attagaggta
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 181 tctgatccgt acagtgattt cgaaaaggtt acaggacgga ttgataagaa tgtatcacca
 241 gaggcaagac acccccttgt ggcagcttat ccgattgtac atgtagatat ggagaatatt
 301 attctctcaa aaaatgagga tcaatccaca cagaatactg atagtgaaac gagaacaata
 361 agtaaaaata cttctacaag taggacacat actagtgaag tacatggaaa tgcagaagtg
 421 catgcgtcgt tctttgatat tggtgggagt gtatctgcag gatttagtaa ttcgaattca
 481 agtacggtcg caattgatca ttcactatct ctagcagggg aaagaacttg ggctgaaaca
 541 atgggtttaa ataccgctga tacagcaaga ttaaatgcca atattagata tgtaaatact
 601 gggacggctc caatctacaa cgtgttacca acgacttcgt tagtgttagg aaaaaatcaa
 661 acactcgcga caattaaagc taaggaaaac caattaagtc aaatacttgc acctaataat
 721 tattatcctt ctaaaaactt ggcgccaatc gcattaaatg cacaagacga tttcagttct
 781 actccaatta caatgaatta caatcaattt cttgagttag aaaaaacgaa acaattaaga
 841 ttagatacgg atcaagtata tgggaatata gcaacataca attttgaaaa tggaagagtg
 901 agggtggata caggctcgaa ctggagtgaa gtgttaccgc aaattcaaga aaca
```

(SEQ ID No 8)

```
   1 SAGPTVPDRD NDGIPDSLEV EGYTVDVKNK RTFLSPWISN IHEKKGLTKY KSSPEKWSTA
  61 SDPYSDFEKV TGRIDKNVSP EARHPLVAAY PIVHVDMENI ILSKNEDQST QNTDSETRTI
 121 SKNTSTSRTH TSEVHGNAEV HASFFDIGGS VSAGFSNSNS STVAIDHSLS LAGERTWAET
 181 MGLNTADTAR LNANIRYVNT GTAPIYNVLP TTSLVLGKNQ TLATIKAKEN QLSQILAPNN
 241 YYPSKNLAPI ALNAQDDFSS TPITMNYNQF LELEKTKQLR LDTDQVYGNI ATYNFENGRV
 301 RVDTGSNWSE VLPQIQETTA RIIFNGKDLN LVERRIAAVN PSDPLETTKP DMTLKEALKI
 361 AFGFNEPNGN LQYQGKDITE FDFNFDQQTS QNIKNQLAEL NATNIYTVLD KIKLNAKMNI
 421 LIRDKR
```

(SEQ ID No 9)

Figure 3. Cont.

```
   1 agtgctggac ctacggttcc agaccgtgac aatgatggaa tccctgattc attagaggta
  61 gaaggatata cggttgatgt caaaaataaa agaactttte tttcaccatg gatttctaat
 121 attcatgaaa agaaaggatt aaccaaatat aaatcatctc ctgaaaaatg gagcacggct
 181 tctgatccgt acagtgattt cgaaaaggtt acaggacgga ttgataagaa tgtatcacca
 241 gaggcaagac acccccttgt ggcagcttat cogattgtac atgtagatat ggagaatatt
 301 attctctcaa aaaatgagga tcaatccaca cagaatactg atagtgaaac gagaacaata
 361 agtaaaaata cttctacaag taggacacat actagtgaag tacatggaaa tgcagaagtg
 421 catgcgtcgt tctttgatat tggtgggagt gtatctgcag gatttagtaa ttcgaattca
 481 agtacggtcg caattgatca ttcactatct ctagcagggg aaagaacttg ggctgaaaca
 541 atgggtttaa ataccgctga tacagcaaga ttaaatgcca atattagata tgtaaatact
 601 gggacggctc caatctacaa cgtgttacca acgacttcgt tagtgttagg aaaaaatcaa
 661 acactcgcga caattaaagc taaggaaaac caattaagtc aaatacttgc acctaataat
 721 tattatcctt ctaaaaactt ggcgccaatc gcattaaatg cacaagacga tttcagttct
 781 actccaatta caatgaatta caatcaattt cttgagttag aaaaaacgaa acaattaaga
 841 ttagatacgg atcaagtata tgggaatata gcaacataca attttgaaaa tggaagagtg
 901 agggtggata caggctcgaa ctggagtgaa gtgttaccgc aaattcaaga aacaactgca
 961 cgtatcattt ttaatggaaa agatttaaat ctggtagaaa ggcggatagc ggcggttaat
1021 cctagtgatc cattagaaac gactaaaccg gatatgacat taaaagaagc ccttaaaata
1081 gcatttggat ttaacgaacc gaatggaaac ttacaatatc aagggaaaga cataaccgaa
1141 tttgatttta atttcgatca acaaacatct caaaatatca agaatcagtt agcggaatta
1201 aacgcaacta acatatatac tgtattagat aaaatcaaat taaatgcaaa aatgaatatt
1261 ttaataagag ataaacgt
```

(SEQ ID No 10)

```
   1 EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL ENIPSENQYF
  61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
 121 QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
 181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
 241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRTHT
 301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GINTADTARL
 361 NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
 421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
 481 LPQIQETTAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL
 541 QYQGKDITEF DFNFDQQTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKR
```

(SEQ ID No 11)

Figure 3 Cont.

```
   1 gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta
  61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
 121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt
 181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
 241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
 301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat
 361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
 421 aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct
 481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
 541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
 601 acttttcttt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa
 661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca
 721 ggacggattg ataagaatgt atcaccagag gcaagacacc ccttgtggc agcttatccg
 781 attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag
 841 aatactgata gtgaaacgag aacaataagt aaaaatactt ctacaagtag gacacatact
 901 agtgaagtac atggaaatgc agaagtgcat gcgtcgttct ttgatattgg tgggagtgta
 961 tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta
1021 gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata ccgctgatac agcaagatta
1081 aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg
1141 acttcgttag tgttaggaaa aaatcaaaca ctcgcgacaa ttaaagctaa ggaaaaccaa
1201 ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca
1261 ttaaatgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt
1321 gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca
1381 acatacaatt ttgaaaatgg aagagtgagg gtggatacag gctcgaactg gagtgaagtg
1441 ttaccgcaaa ttcaagaaac aactgcacgt atcattttta atggaaaaga tttaaatctg
1501 gtagaaaggc ggatagcggc ggttaatcct agtgatccat tagaaacgac taaaccggat
1561 atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaactta
1621 caatatcaag ggaaagacat aaccgaattt gattttaatt tcgatcaaca aacatctcaa
1681 aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa
1741 atcaaattaa atgcaaaaat gaatatttta ataagagata aacgt
```

(SEQ ID No 12)

```
   1 EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL ENIPSENQYF
  61 QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
 121 QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN
 181 DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
 241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSQTRTIS KNTSTSRTHT
 301 SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL
 361 NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA
 421 LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
 481 LPQIQETTAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL
 541 QYQGKDITEF DFNFDQQTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
 601 NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS GYIVEIEDTE GLKEVINDRY
 661 DMLNISSLRQ DGKTFIDFKK YNDKLPLYIS NPNYKVNVYA VTKENTIINP SENGDTSTNG
 721 IKKILIFSKK GYEIG
```

(SEQ ID No 13)

Figure 3 Cont.

```
   1 gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta
  61 ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca
 121 ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt
 181 caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct
 241 acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct
 301 tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat
 361 caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa
 421 aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct
 481 tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat
 541 gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga
 601 acttttcttt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa
 661 tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca
 721 ggacggattg ataagaatgt atcaccagag gcaagacacc cccttgtggc agcttatccg
 781 attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag
 841 aatactgata gtgaaacgag aacaataagt aaaaatactt ctacaagtag gacacatact
 901 agtgaagtac atggaaatgc agaagtgcat gcgtcgttct ttgatattgg tgggagtgta
 961 tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta
1021 gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata ccgctgatac agcaagatta
1081 aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg
1141 acttcgttag tgttaggaaa aaatcaaaca ctcgcgacaa ttaaagctaa ggaaaaccaa
1201 ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca
1261 ttaaatgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt
1321 gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca
1381 acatacaatt ttgaaaatgg aagagtgagg gtggatacag gctcgaactg gagtgaagtg
1441 ttaccgcaaa ttcaagaaac aactgcacgt atcatttta atggaaaaga tttaaatctg
1501 gtagaaaggc ggatagcggc ggttaatcct agtgatccat tagaaacgac taaaccggat
1561 atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaactta
1621 caatatcaag ggaaagacat aaccgaattt gattttaatt tcgatcaaca aacatctcaa
1681 aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa
1741 atcaaattaa atgcaaaaat gaatatttta ataagagata aacgttttca ttatgataga
1801 aataacatag cagttggggc ggatgagtca gtagttaagg aggctcatag agaagtaatt
1861 aattcgtcaa cagagggatt attgttaaat attgataagg atataagaaa aatattatca
1921 ggttatattg tagaaattga agatactgaa gggcttaaag aagttataaa tgacagatat
1981 gatatgttga atatttctag tttacggcaa gatggaaaaa catttatagg ttttaaaaaa
2041 tataatgata aattaccgtt atatataagt aatcccaatt ataaggtaaa tgtatatgct
2101 gttactaaag aaaacactat tattaatcct agtgagaatg gggatactag taccaacggg
2161 atcaagaaaa ttttaatctt ttctaaaaaa ggctatgaga taggataa
```

(SEQ ID No 14)

Figure 3 Cont.

```
  1 FHYDRNNIAV GADESVVKEA HREVINSSTE GLLLNIDKDI RKILSGYIVE IEDTEGLKEV
 61 INDRYDMLNI SSLRQDGKTF IDFKKYNDKL PLYISNPNYK VNVYAVTKEN TIINPSENGD
121 TSTNGIKKIL IFSKKGYEIG
```

(SEQ ID No 15)

```
  1 tttcattatg atagaaataa catagcagtt ggggcggatg agtcagtagt taaggaggct
 61 catagagaag taattaattc gtcaacagag ggattattgt taaatattga taaggatata
121 agaaaaatat tatcaggtta tattgtagaa attgaagata ctgaagggct taaagaagtt
181 ataaatgaca gatatgatat gttgaatatt tctagtttac ggcaagatgg aaaaacattt
241 atagatttta aaaaatataa tgataaatta ccgttatata taagtaatcc caattataag
301 gtaaatgtat atgctgttac taaagaaaac actattatta atcctagtga gaatggggat
361 actagtacca acgggatcaa gaaaatttta atcttttcta aaaaaggcta tgagatagga
421 taa
```

(SEQ ID No 16)

Figure 4

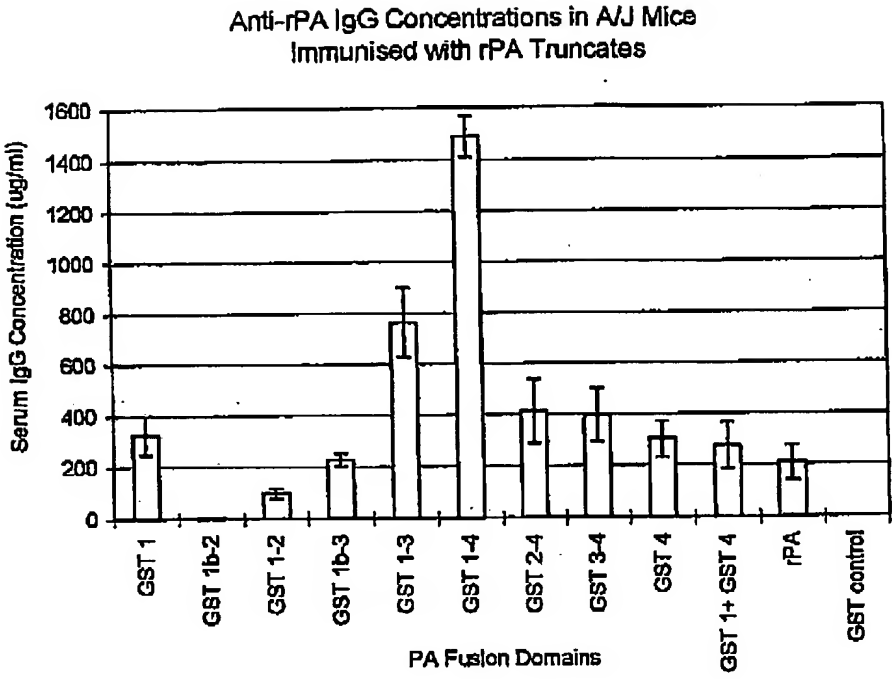
Anti-rPA IgG Concentrations in A/J Mice
Immunised with rPA Truncates
Serum IgG Concentration (ug/ml)
1600
1400
1200
1000
800
600
400
200
0
GST 1
GST 1b-2
GST 1-2
GST 1b-3
GST 1-3
GST 1-4
GST 2-4
GST 3-4
GST 4
GST 1+ GST 4
rPA
GST control
PA Fusion Domains

Figure 5

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/GB 01/03065

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/70 C07K14/32 A61K39/07 C12N15/31 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROSSIER FABIEN ET AL: "Role of toxin functional domains in anthrax pathogenesis." INFECTION AND IMMUNITY, vol. 68, no. 4, April 2000 (2000-04), pages 1781-1786, XP002183267 ISSN: 0019-9567 figure 1 page 1785, left-hand column, last paragraph -right-hand column -/--	1-23, 25-31, 34,35

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2001	30/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mata Vicente, T.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 01/03065

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	FLICK-SMITH H C ET AL: "Protective efficacy of PA domains in mice." IMMUNOLOGY, vol. 101, no. Supplement 1, December 2000 (2000-12), page 83 XP002183268 Annual Congress of the British Society for Immunology;Harrogate, UK; December 05-08, 2000 ISSN: 0019-2805 the whole document	1-23, 25-31, 34,35
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Inte nal Application No
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